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Cytomegalovirus Nucleic Acid Distribution Within the Human Vascular Tree

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The presence of human cytomegalovirus (HCMV) nucleic acids was demonstrated in almost all major arteries of HCMV-seropositive patients by polymerase chain reaction. The amount of HCMV nucleic acids present in the arterial wall was calculated to be less than 2 viral genomic equivalents per 2500 human genomic equivalents. No relation was observed between the presence of HCMV nucleic acids in the arterial wall and the existence of atherosclerotic changes. No differences were observed in the number of viral genomic equivalents present at sites with or without atherosclerosis. Apart from a role for HCMV in the pathogenesis of atherosclerosis, the presence of latent HCMV in the arterial tree provides a powerful system to describe the physical state of this latently present virus. (Am J Pathol 1991, 138:563–567)

Infections with human cytomegalovirus (HCMV) are common, and a prominent characteristic feature of this virus is that it persists in the host after a primary infection. Frequent reactivation from this latent state has been documented by various authors.[1]

Recently several reports suggested that the arterial wall might be a site of latency for this virus[2,3] and it was suggested that frequent reactivation from this latent state might contribute significantly to the pathogenesis of atherosclerosis.[2–7]

In our laboratory, we could demonstrate by dot-blot and *in situ* DNA hybridization, and by polymerase chain reaction (PCR),[3,5] that the complete viral genome was present in a latent form in a high percentage of arterial wall specimens obtained from proximal elastic arteries. We were also able to show by PCR that the prevalence of CMV nucleic acids was much higher in samples obtained from patients with grade III atherosclerosis (90%) as compared with samples obtained from matched controls with maximally grade I atherosclerosis (50%). These findings do indeed suggest a role for this virus in the pathogenesis of atherosclerosis. Most arterial samples, however, were taken from arteries prone to develop atherosclerosis. Various autopsy series clearly demonstrated that atherosclerosis is not a general disease of all the arteries but that there are several predetection sites in the human arterial tree at risk to develop lesions. These studies demonstrated a high prevalence of atherosclerotic lesions in abdominal aorta, carotid artery, common iliac artery and left anterior descending artery but showed a low prevalence in celiac and pulmonal arteries. Considering a role for CMV in the pathogenesis of atherosclerosis, one may suggest a tropism from this virus for these predilection sites or that simply more viral nucleic acids will accumulate at these arterial sites as compared with the nonatherosclerotic sites.

In this report we describe the distribution of CMV nucleic acids over the human arterial tree in a qualitative way as determined by polymerase chain reaction, and in a semiquantitative way as determined by dot-blot DNA hybridization.

Materials and Methods

Tissue Specimens

The subjects of this study consisted of HCMV-seropositive patients on whom autopsy was performed within 12 hours after death. Seropositivity for HCMV was confirmed by using a latex agglutination test (CMV scan, Beckton & Dickinson, Oxford, UK).

Only patients with non–CMV-related disease were included in this study. Patients suffering from immunosuppressive disease or undergoing immunosuppressive therapy were strictly excluded. The possibility of active CMV infection or reactivation of latently present virus was ruled out by testing for the presence of gamma M immunoglobulin (IgM) and IgA anti-CMV antibodies using a capture technique.[8] At autopsy, samples were taken

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from all major elastic arteries (Tables 1, 2) and from the portal vein.

Processing of Tissue Specimen

Tissue specimen for *in situ* DNA hybridization were formalin fixed and paraffin embedded using routine histologic procedures. For PCR and semiquantitative dot-blot, DNA was extracted from the tissue specimen by proteolytic digestion and phenol-chloroform extraction followed by ethanol precipitation.[5]

Microscopy

All sections were routinely stained with hematoxylin and eosin (H&E) and investigated for the presence of atherosclerosis. Only vessel walls with minimal intimal thickening and no signs of atheroma formation were considered to be free of atherosclerosis.

Oligomer Synthesis

The oligomers used as primers in the PCR were synthesized on a DNA synthesizer (cyclone DNA synthesizer, Biosearch Inc., New Brunswick, NJ) by the fosfamide method. The sequences for the oligomer set were reactive with the major sequences described by Akrigg et al[9] and located in the ES fragment used as a probe for the DNA hybridization techniques. The sequences are: 5^1-GGA-GAT-GTG-GAT-GGC-TTG-TA-3^1 (IE_1 upstream primer) and 5^1-GGA-GGC-TGA-GTT-CTT-GGT-AA-3^1 (IE_2 downstream primer). The oligomer set reactive with the human β-globine gene has been described elsewhere.[10] The sizes of the PCR amplification products of the IE and the β-globin targets are 170 and 110 base pairs, respectively. The specificity and sensitivity of both primer sets have been tested extensively.[3,10]

Polymerase Chain Reaction

The polymerase chain reaction protocol performed on extracted cellular DNA has been described previously[3] and was performed on a premIII (LEP Scientific, Buck, UK). After a total of 35 cycles of amplification, 10 μl of the reaction mixture was subjected to electrophoresis on 2% agarose gels (Seakem GTG FMC Bioproducts, Rockland, ME) containing 0.5 μg/ml ethidium bromide. The amplification product was visualized by ultraviolet fluorescence. DNA samples that did not show reactivity with the CMV primers were further analyzed with the primer set reactive with the β-globin gene to check on DNA preservation.

Hybridization Probes

DNA hybridization was performed with the 7.0-Kb ES fragment,[3,5] an $EcoR_1$-SalI subdigestion of the $EcoR_1$-J fragment of CMV strain AD169. Controls consisted of total HCMV strain AD169 DNA, total rat cytomegalovirus (RCMV) DNA,[11] and plasmid pBR328 DNA without inserts. In all, DNA biotin was inserted by incorporation of biotin-11-dUTP (Bethesda Research Laboratories), using a random primed DNA labeling kit (Boehringer-Mannheim, Indianapolis, IN).

Semiquantitative Dot-blot DNA Hybridization

Spotting of cellular DNA on nitrocellulose paper, hybridization conditions, and visualization of successful hybrid-

Table 1. *Polymerase Chain Reaction and Stratification for Atherosclerosis*

Vessel	PCR (total) pos/total (%)	Atherosclerosis pos/total (%)	PCR + and atherosclerosis +	PCR + and atherosclerosis −
Thoracic aorta	8/9	6/9	5/6	3/3
Abdominal aorta	8/9	8/9	8/8	0/1
Carotid artery	8/9	7/9	7/7	1/2
Superior mesenteric artery	8/9	8/9	7/8	1/1
Celiac artery	7/7	6/7	6/6	1/1
Renal artery	9/9	7/9	7/7	2/2
Common iliac artery	9/9	6/9	6/6	3/3
Pulmonal artery	7/7	1/7	1/1	6/6
Left anterior descending artery	8/9	9/9	8/9	0/0
Portal vein	8/8	NR	NR	NR
Total arteries (except portal vein)	72/77 (94)	58/77 (75)	55/58 (95)	17/19 (90)

NR, not relevant.

Table 2. *Dot-blot DNA Hybridization and Stratification for Atherosclerosis*

Vessel	Dot-blot (total) pos/total (%)	Atherosclerosis pos/total (%)	Dot-blot + and atherosclerosis +	Dot-blot + and atherosclerosis −
Thoracic aorta	8/9	6/9	5/6	3/3 (100)
Abdominal aorta	4/9	8/9	4/8	0/1 (−)
Carotid artery	5/9	7/9	4/7	1/2 (50)
Superior mesenteric artery	7/9	8/9	6/8	1/1 (100)
Celiac artery	5/7	6/7	4/6	1/1 (100)
Renal artery	9/9	7/9	7/7	2/2 (100)
Common iliac artery	7/9	6/9	5/6	2/3 (67)
Pulmonal artery	5/7	1/7	0/1	5/6 (83)
Left anterior descending artery	8/9	9/9	8/9	0/0 (−)
Portal vein	6/8	NR	NR	NR
Total arteries (except portal vein)	58/77 (75)	58/77 (75)	43/58 (73)	15/19 (83)

NR, not relevant.

ization was carried out as described previously.[3,5] The specificity of the biotinylated probes has been evaluated extensively.[3,5] Each arterial DNA sample was serially diluted to produce spots of 1, 0.5, 0.25, and 0.12 μg DNA, respectively. Controls consisted of purified HCMV DNA and DNA extracted from noninfected human embryonic fibroblasts (HEF) (Flow 2002, Flow Laboratories, McClean, VA). To estimate the amount of viral DNA present in the arterial DNA samples, spots were produced of 1, 0.5, 0.25, 0.12, 0.06, and 0.03 picogram purified HCMV DNA made up to a total of 0.5 μg DNA per spot with DNA extracted from noninfected HEF. In all experiments, 0.1 pg of purified HCMV DNA could be detected, equaling approximately 350 viral genomic equivalents (VGE) (MW $= 1.7 \times 10^8$ dalton). Estimating the total DNA content of one cell nucleus to be 6.6×10^{11} dalton, it is possible to calculate the amount of VGE present per human genomic equivalent (HGE).

In Situ *DNA Hybridization*

The *in situ* DNA hybridization protocol performed on 4-μ–thick tissue sections has been described previously.[5] The specificity of the biotinylated probes has been evaluated extensively.[5]

Statistical Analysis

Statistical analysis was carried out using Fisher's exact tests. Results were considered significant when $P < 0.01$.

Results

Tissue Specimen

Nine autopsy patients (five men, four women) with a mean age of 74 years (range, 47 to 92 years) were available. Three men and four women died of metastatic neoplastic disease (colon carcinoma: 3; breast carcinoma: 3; thyroid carcinoma: 1) and two men died of cardiac arrest. All subjects available for this study were shown to contain anti-HCMV antibodies of the IgG class, but not of the IgM or IgA classes, which excluded primary and frequently reactivating disease. These nine autopsy patients provided 77 arterial samples.

Microscopy

From all arterial samples, 58 of 77 (75%) showed clear signs of atherosclerosis with marked intimal thickening and sometimes atheroma formation. Only the pulmonal artery was almost always free of atherosclerotic changes (Table 1). Only in one of seven (14%), was slight intimal thickening observed. Each individual patient showed atherosclerotic disease of six or more main vessels, but also had arteries free of intimal thickening, making this group of patients suitable for this study.

Polymerase Chain Reaction

The polymerase chain reaction (PCR) performed on extracted DNA from all major arteries (Table 1) showed that 72 of 77 (94%) samples contained HCMV nucleic acids. These nucleic acids were equally distributed over the vascular tree.

There was no difference observed in the prevalence of HCMV DNA in samples obtained from arteries with atherosclerotic changes as compared with samples obtained from microscopically normal arterial walls ($P = 0.36$).

Samples negative for HCMV nucleic acids by PCR (5 of 77 [6%]) were checked for DNA integrety with a primer set reactive with the human β-globin gene. All samples

tested with this primer set were positive for the presence of the β-globin gene, proving that the DNA integrety was sufficient for PCR analysis.

Semiquantitative Dot-blot DNA Hybridization

Using the dot-blot DNA hybridization technique (Table 2), HCMV nucleic acid sequences were detected in 58 of 77 (75%) samples. Human CMV DNA was detected in 43 of 58 (73%) samples obtained from atherosclerotic arteries and in 15 of 18 (83%) samples obtained from normal arteries. Statistical analysis showed this difference to not be significant ($P = 0.46$). Most of the samples containing detectable amounts of HCMV nucleic acids only showed positive hybridization in the less diluted DNA samples, meaning that 0.1 to 0.2 pg of viral DNA was present in 1 μg DNA extracted from arterial walls. From this we concluded that approximately one to two viral genomic equivalents (VGE) were present per 2500 human genomic equivalents (HGE). Only four samples, all obtained from one patient, could be diluted fourfold and still show positive hybridization. This means that in these samples approximately one to two VGE were present per 600 HGE. There was no difference observed in the number of VGE per HGE present in arteries with or without atherosclerotic changes.

All samples negative with the dot-blot DNA hybridization technique but positive by PCR should contain less than 1 VGE per 2500 HGE. *In situ* DNA hybridization of sections of arteries with atherosclerotic changes showed that 24 of 58 (41%) contained HCMV nucleic acid sequences, whereas sections of arteries without these changes showed the presence of nucleic acids in 7 of 19 (37%), which was not statistically different.

In Situ *DNA Hybridization*

Histologically the HCMV nucleic acid sequences detected with the *in situ* DNA hybridization technique were mostly located in apparently normal arterial tissues without atherosclerotic changes. Only in a few occasions the viral nucleic acids were located in the thickened intimal layer. No HCMV nucleic acid sequences were detected in sites of atheroma formation. Sequential sections stained with H&E showed that the DNA hybridization reactivity mostly was located in smooth muscle cells in the arterial media.

Discussion

In this study we have demonstrated by PCR that probably all major arteries obtained from HCMV-seropositive patients contain HCMV nucleic acid sequences. These nucleic acids are equally distributed among arteries with and without atherosclerotic changes. By using semiquantitative dot-blot DNA hybridization techniques, it was possible to estimate that approximately 2 or less VGE are present per 2500 HGE.

There were no differences observed in the number of VGE per HGE present in arteries with or without atherosclerotic changes. Human CMV nucleic acids were mostly located in smooth muscle cells of the arterial media, as shown by *in situ* DNA hybridization.

One of the objects of this study was to see if HCMV nucleic acids preferentially accumulated at arterial sites prone to develop atherosclerotic lesions. The finding that almost all arteries contain HCMV nucleic acids and that there is no correlation with atherosclerotic sites clearly shows that there is no tropism from the HCMV for these predilection sites. These results could more or less be predicted from previous reports demonstrating the presence of HCMV in various arteries from patients with severe atherosclerosis but also in abdominal aortas without atherosclerotic changes.[2,4–6] Also our previous findings using PCR[3] and demonstrating a high prevalence of HCMV nucleic acids in patients with grade III atherosclerosis (90%) but also in patients with maximally grade I atherosclerosis (50%) did suggest these results. As already stated,[3,5] we do not suggest a direct role for HCMV in the pathogenesis of atherosclerosis.

The finding that arteries with or without atherosclerotic changes contain approximately equal numbers of VGE per HGE also means that there is no direct relationship between the HCMV and the presence of atherosclerosis and again a possible reason for these findings is that the vessels under investigation represent end-stage atherosclerotic changes not influenced any more by the HCMV. The role for HCMV in the pathogenesis of atherosclerosis might be at the start of this disease with the development of fatty streaks.[12,13] Considering the tropism from HCMV for endothelial cells,[14] infection of these cells could result in the production of inflammatory mediators such as interleukin-I, which subsequently induce activation and adherence of leukocytes responsible for injury of the endothelium[13] being the initial process in the response to injury theory of Ross.[15] This injury, in cooperation with other established risk factors, could start the process of atherogenesis. Once present in the arterial wall, the HCMV will remain latently present for the rest of life. Transient local reactivation from this latent state[16] might also contribute further to the process of atherogenesis.

Apart from the process of atherogenesis, the presence of latent HCMV in the human arterial wall remains an intriguing phenomenon. Considering the wide tropism of this virus for many different tissues during reproductive infection,[17] one might ask if the human vascular wall is the

only site of latency. Probably more cell types will contain latent HCMV. Our finding that at most two VGE are present per 2500 HGE may substantiate this probability because these low numbers were until recently beyond the reach of molecular biology.

Comparable viruses like herpes simplex virus (HSV) were found to be present at at least 1 VGE per 100 HCE in trigeminal ganglia,[18] facilitating their detection. Objects for further research are therefore obvious: at first screening other tissues on the presence of latent HCMV, and secondly, characterization of the physical state of this virus at least latently present in the human arterial tree.

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